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SPECIFICATION FOR BONE-MEAL AS LIVESTOCK FEED SUPPLEMENT

(First Revision)

Second Reprint MAY 1985
(Incorporating Amendment No. 1)

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(*Cover page and page* 1, *Title*) — Substitute the following for the existing title:

'SPECIFICATION FOR BONE-MEAL AS POULTRY FEED SUPPLEMENT'

(FAD 5)

Reprography Unit, BIS, New Delhi, India

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[*Cover page and page* 1, *Title* (*see also Amendment No.* 1)] — Substitute the following for the existing title:

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(*Page* 3, *clause* **0.2**, *line* 1) — Substitute 'poultry' *for* 'livestock' and wherever the word 'livestock' appears in the standard.

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Indian Standard

SPECIFICATION FOR BONE-MEAL AS LIVESTOCK FEED SUPPLEMENT

(First Revision)

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Indian Standard

SPECIFICATION FOR BONE-MEAL AS LIVESTOCK FEED SUPPLEMENT

(First Revision)

0. FOREWORD

0.1 This Indian Standard (First Revision) was adopted by the Indian Standards Institution on 3 April 1968, after the draft finalized by the Animal Feeds Sectional Committee had been approved by the Agricultural and Food Products Division Council.

0.2 Bone-meal is used as a mineral supplement in livestock feeds; its chief mineral constituents being calcium and phosphorus. The starting material is undecomposed bones and these sometimes get contaminated with pathogenic organisms like *Bacillus anthracis*, *Clostridium botulinum*, *Clostridium chauvaei* and *Clostridium septicum*. It is essential, therefore, that the bone-meal meant for use in livestock feeds is free from these organisms. In order to ensure that the bone-meal is safe for feeding the livestock, absence of the spores of these organisms has been included as one of the requirements in this standard.

0.2.1 This standard was first issued in 1961. In view of the changed raw material conditions, that is, change in the composition of bones of animals due to low level of nutrition or other agro-climatic factors, and to accommodate the varying processes employed for producing feed grade bone-meal this revision is being issued. In this revised standard, the limits for various characteristics have been reconsidered and revised and a limit for the characteristic total ash has been included.

0.3 Bone-meal meant for livestock feeding is prepared by defatting and sterilizing undecomposed bones by steam under pressure. The sterilized bones are then dried and ground to the required fineness.

0.4 For the purpose of deciding whether a particular requirement of this standard is complied with, the final value, observed or calculated, expressing the result of a test or analysis, shall be rounded off in accordance with IS : 2-1960*. The number of significant places retained in the rounded off value should be the same as that of the specified value in this standard.

---

*Rules for rounding off numerical values (*revised*).

1. SCOPE

1.1 This standard prescribes the requirements and the methods of sampling and test for bone-meal to be used as a mineral supplement in livestock feeds.

2. REQUIREMENTS

2.1 Description — Bone-meal shall be obtained from undecomposed bones after removal of adhering tissues, defatting, sterilization by steam under pressure, drying and grinding. The material shall be nearly white in colour and the particle size shall be such that not less than 90 percent by mass of the material passes through 106-micron IS Sieve (*see* IS : 460-1962*). Bone-meal shall not smell even after prolonged storage and shall be free from adulterants, insect and fungus infestation.

2.2 Bone-meal shall also conform to the requirements prescribed in Table 1.

TABLE 1 REQUIREMENTS FOR BONE-MEAL AS LIVESTOCK FEED SUPPLEMENT

SL No.	CHARACTERISTIC	REQUIREMENT	METHOD OF TEST (REF TO APPENDIX)
(1)	(2)	(3)	(4)
i)	Moisture, percent by mass, *Max*	7	A
ii)	Calcium, percent by mass, *Min*	31	B
iii)	Phosphorus, percent by mass, *Min*	14	C
iv)	Crude fat, percent by mass, *Max*	1·0	D
v)	Total ash, percent by mass, *Min*	85	E
vi)	Acid insoluble ash, percent by mass, *Max*	1·0	F
vii)	Fluorine, percent by mass, *Max*	0·60	G
viii)	Spores of *Bacillus anthracis* and *Clostridium* sp.	Nil	H

NOTE — The values specified for requirements (ii) to (vii) are on moisture-free basis.

3. PACKING AND MARKING

3.1 Packing — Bone-meal shall be packed in moisture-proof bags or in any other suitable container. The containers used shall be sound, clean and free from causal agents of infectious diseases and parasites.

3.2 Marking — Each container shall be suitably marked or labelled and shall give the following information:

a) Name of the material,
b) Name of the manufacturer,
c) Batch or code number,
d) Net mass,
e) Date of packing, and
f) Guaranteed analysis.

---

*Specification for test sieves (*revised*).

3.2.1 Each container may also be marked with the ISI Certification Mark.

NOTE — The use of the ISI Certification Mark is governed by the provisions of the Indian Standards Institution (Certification Marks) Act and the Rules and Regulations made thereunder. The ISI Mark on products covered by an Indian Standard conveys the assurance that they have been produced to comply with the requirements of that standard under a well-defined system of inspection, testing and quality control which is devised and supervised by ISI and operated by the producer. ISI marked products are also continuously checked by ISI for conformity to that standard as a further safeguard. Details of conditions under which a licence for the use of the ISI Certification Mark may be granted to manufacturers or processors, may be obtained from the Indian Standards Institution.

4. SAMPLING

4.1 Representative samples of the material for testing conformity to this specification shall be drawn according to the method prescribed in Appendix J.

5. TESTS

5.1 Tests shall be carried out as prescribed in the relevant appendices specified in col 4 of Table 1.

5.2 Quality of Reagents — Unless specified otherwise, pure chemicals and distilled water (*see* IS : 1070-1977*) shall be employed in all tests.

NOTE — 'Pure chemicals' shall mean chemicals that do not contain impurities which affect the experimental results.

APPENDIX A

[*Table* 1, *Item* (i)]

DETERMINATION OF MOISTURE

A-1. PROCEDURE

A-1.1 Weigh accurately about 5 g of the material in an aluminium dish having a diameter of at least 50 mm and a depth not exceeding 40 mm previously dried in an oven and weighed. Shake the dish until the contents are evenly distributed. With the cover removed, place the dish in an air-oven maintained at 100 ± 2°C for four hours to dry. Cool in a desiccator and weigh. Repeat the process of heating, cooling and weighing till the difference in mass between two successive weighings is less than one milligram. Record the lowest mass. Reserve the dried material for the determination of acid insoluble ash (*see* **E-2.1**).

---

*Specification for water for general laboratory use (*second revision*).

A-2. CALCULATION

A-2.1 Moisture, percent by mass $= \frac{100 (W_1 - W_2)}{W_1 - W}$

where

W_1 = mass in g of the dish with the material before drying,
W_2 = mass in g of the dish with the material after drying, and
W = mass in g of the empty dish.

APPENDIX B

[*Table* 1, *Item* (ii)]

DETERMINATION OF CALCIUM

B-1. REAGENTS

B-1.1 Hydrochloric Acid — 25 ml of concentrated hydrochloric acid (*see* IS : 265-1950*) diluted to 100 ml.

B-1.2 Methyl Red Indicator — Dissolve 0·15 g of methyl red in 500 ml of water.

B-1.3 Ammonium Hydroxide Solution — 50 percent (*v/v*).

B-1.4 Dilute Ammonium Hydroxide Solution — 2 percent (*v/v*).

B-1.5 Ammonium Oxalate Solution — saturated.

B-1.6 Concentrated Sulphuric Acid — sp gr 1·84 (*see* IS : 266-1961†).

B-1.7 Standard Potassium Permanganate Solution — 0·1 N (*see* IS : 2316-1963‡).

B-2. PROCEDURE

B-2.1 Preparation of the Solution — Weigh accurately about one gram of the material in a silica basin and ignite at 600 ± 20°C in a muffle furnace to carbon-free ash. Boil the ash in 40 ml of hydrochloric acid and a few drops of nitric acid. Transfer to a 250-ml graduated flask. Cool, dilute to the mark and mix thoroughly and filter through a dry fluted filter paper. The clear solution is to be used also for the determination of phosphorus (*see* **C-2.1**).

---

*Specification for hydrochloric acid. (Since revised).

†Specification for sulphuric acid (*revised*).

‡Methods of preparation of standard solutions for colorimetric and volumetric analysis (Since revised).

B-2.2 Transfer a 25-ml aliquot of the solution prepared as in **B-2.1** to a 400-ml beaker, dilute to about 100 ml with water and add two drops of methyl red indicator solution. Add ammonium hydroxide solution dropwise till a brownish-orange colour is obtained (*p*H 5·6). Add two drops of hydrochloric acid so that the colour of the solution is pink (*p*H 2·5 to 3·0). Dilute to about 150 ml, bring to the boil and add slowly, with constant stirring, 10 ml of hot ammonium oxalate solution. If the red colour of the solution changes to orange or yellow, add hydrochloric acid dropwise until the colour again changes to pink. Leave overnight to allow the precipitate to settle. Filter the supernatant liquid through an ashless filter paper and wash the precipitate thoroughly with dilute ammonium hydroxide solution. Place the paper with the precipitate in the beaker in which precipitation was carried out and add a mixture of 125 ml of water and 5 ml of concentrated sulphuric acid. Heat to a temperature between 70°C and 90°C, and titrate with the standard potassium permanganate solution until the first slight pink colour is obtained.

B-3. CALCULATION

B-3.1 Calcium (on moisture-free basis), percent by mass $= \frac{2\,000\, AN}{W(100 - M)}$

where

A = volume in ml of the standard potassium permanganate solution required in the titration;

N = normality of the standard potassium permanganate solution;

W = mass in g of the material taken for the test (*see* **B-2.1**); and

M = moisture, percent by mass (*see* **A-2.1**).

APPENDIX C

[*Table* 1, *Item* (iii)]

DETERMINATION OF PHOSPHORUS

C-1. REAGENTS

C-1.1 Concentrated Nitric Acid — sp gr 1·42 (*see* IS : 264-1950*).

C-1.2 Nitric Acid (1 : 1) — mixture of equal volumes of concentrated nitric acid and water.

---

*Specification for nitric acid (Since revised).

C-1.3 Ammonium Molybdate Stock Solution — Take 200 g of powdered ammonium molybdate in a stoppered graduated cylinder of 1 000 ml capacity, add to it 800 ml of water and shake well for 25 minutes to dissolve the ammonium molybdate. Add gradually 25 percent (*m/v*) ammonium hydroxide solution till the solution is clear (about 100 to 140 ml of ammonium hydroxide may be required). Avoid adding excess of ammonia. Make up the volume to one litre. If necessary, filter the solution through a fluted filter paper and stock this solution.

C-1.4 Dilute Nitric Acid — 2 percent (*m/v*).

C-1.5 Potassium Nitrate Solution — 3 percent (*m/v*).

C-1.6 Standard Sodium Hydroxide Solution — 0·1 N.

C-1.7 Standard Nitric Acid — 0·1 N.

C-1.8 Phenolphthalein Indicator Solution — Dissolve 0·1 g of phenolphthalein in 100 ml of 60 percent (*m/v*) rectified spirit (*see* IS : 323-1959*).

C-2. PROCEDURE

C-2.1 Precipitation — Take a 10-ml aliquot of the prepared solution (*see* **B-2.1**) in a 150-ml beaker. In a dry beaker, prepare ammonium molybdate solution by pouring into it, quickly and simultaneously 10 ml of the ammonium molybdate stock solution (*see* **C-1.3**) and 10 ml of concentrated nitric acid; or take 10 ml of the concentrated nitric acid first in the beaker and into this pour quickly 10 ml of the ammonium molybdate stock solution, whirling the beaker during addition. Pour this freshly prepared, clear liquid into the beaker containing the aliquot and stir.

NOTE — The temperature developed in the molybdate solution is sufficient to precipitate all the phosphorus present in the aliquot. Under no circumstances the phosphomolybdate precipitate should be heated either on a water-bath or directly over a burner so as to avoid precipitation of molybdic anhydride.

C-2.2 Filtration and Washing — Allow the precipitate to stand for 15 hours and then filter through a disc of Whatman filter paper No. 42 in a Gooch crucible by suction or through a 9-cm Whatman filter paper No. 42 over an ordinary funnel. As far as possible, only the supernatant liquid is passed through the filter paper, retaining the precipitate in the beaker. When the supernatant liquid is decanted off, the precipitate is washed twice with dilute nitric acid and then with potassium nitrate solution until the washings are free from acid. If ordinary funnel and filter paper are used, freedom from acidity may be tested by collecting sufficient filtrate in a test-tube to which a few drops of phenolphthalein indicator solution and one drop of the standard sodium hydroxide solution are added. If the pink colour appears with one drop of the standard alkali, the precipitate is free from acid.

---

*Specification for rectified spirit (*revised*).

C-2.3 Titration — Transfer the precipitate with the filter paper back to the beaker in which the precipitation was carried out. When Gooch crucible is used for filtration, transfer the whole crucible along with the filter paper to the beaker in which precipitation was carried out. Add sufficient quantity of the standard sodium hydroxide solution from a burette just sufficient to dissolve the precipitate and then add 5 ml in excess. See that no yellow precipitate sticks to the filter paper. Note the total volume of the standard sodium hydroxide solution added. Add about 10 drops of phenolphthalein indicator solution and titrate the excess of alkali with the standard nitric acid.

C-3. CALCULATION

C-3.1 Phosphorus (on moisture-free basis), percent by mass $= \dfrac{336{\cdot}75\,(AN_1 - BN_2)}{W\,(100 - M)}$

where

A = volume in ml of the standard sodium hydroxide solution used (*see* **C-2.3**);

N_1 = normality of the standard sodium hydroxide solution;

B = volume in ml of the standard nitric acid used to neutralize the excess alkali (*see* **C-2.3**);

N_2 = normality of the standard nitric acid;

W = mass in g of the material taken for the test (*see* **B-2.1**); and

M = moisture, percent by mass (*see* **A-2.1**).

APPENDIX D

[*Table* 1, *Item* (iv)]

DETERMINATION OF CRUDE FAT

D-1. REAGENT

D-1.1 Petroleum Ether — of boiling point 40 to 60°C.

D-2. PROCEDURE

D-2.1 Weigh accurately about 5 g of the sample dried as described under **A-1.1** and extract with petroleum ether for 8 hours in a Soxhlet or other suitable extractor. Dry the extract on a steam-bath for 30 minutes, cool in a desiccator and weigh. Continue at 30-minute intervals, this alternate drying and weighing until the difference in weight between two successive weighings is less than one milligram. Note the lowest mass.

D-3. CALCULATION

D-3.1 Crude fat on ether extract (on moisture-free basis), percent by mass $= \frac{100 (W_1 - W_2)}{W}$

where

W_1 = mass in g of the extraction flask with the dried ether extract,

W_2 = mass in g of the extraction flask, and

W = initial mass in g of the dried material taken for the test.

APPENDIX E

[*Table* 1, *Item* (v)]

DETERMINATION OF TOTAL ASH

E-1. PROCEDURE

E-1.1 Weigh accurately about 2 g of the dried material (*see* **A-2.1**) in a tared procelain, silica or platinum dish. Ignite with the flame of a Meker burner for about one hour. Complete the ignition by keeping in a muffle furnace at 600 ± 20°C until grey ash results. Cool in a desiccator and weigh. Ignite the dish again in the muffle furnace for 30 minutes, cool and weigh. Repeat this process until the difference in weight between two successive weighings is less than 1 mg. Note the lowest mass.

NOTE — Reserve the dish containing this ash for the determination of acid insoluble ash (*see* **F-2**).

E-2. CALCULATION

E-2.1 Total ash (on moisture-free basis), percent by mass $= \frac{100 (W_2 - W)}{W_1 - W}$

where

W_2 = the lowest mass in g of the dish with the ash,

W = mass in g of the empty dish, and

W_1 = mass in g of the dish with the dried material taken for the test.

APPENDIX F

[*Table* 1, *Item* (vi)]

DETERMINATION OF ACID INSOLUBLE ASH

F-1. REAGENT

F-1.1 Dilute Hydrochloric Acid — approximately 5 N, prepared from concentrated hydrochloric acid (*see* IS : 265-1962*).

F-2. PROCEDURE

F-2.1 Weigh accurately about 2 g of the dried material (*see* **A-1.1**) in a tared porcelain, silica or platinum dish. Ignite with a Meker burner for about one hour. Complete the ignition by keeping in a muffle furnace at 500 ± 20°C until grey ash results. Moisten with concentrated hydrochloric acid and evaporate to dryness. Keep in an electric air-oven maintained at 135 ± 2°C for about 3 hours. Cool and add 25 ml of dilute hydrochloric acid, cover with a watch-glass and heat on a water-bath for 10 minutes. Cool and filter through Whatman filter paper No. 42 or its equivalent. Wash the residue with hot water until the washings are free from chlorides as tested with silver nitrate solution and return the filter paper and residue to the dish. Ignite it in a muffle furnace at 500 ± 20°C for one hour. Cool in a desiccator and weigh. Ignite the dish again for 30 minutes, cool and weigh. Repeat this process till the difference between two successive weighings is less than one milligram. Note the lowest mass.

F-3. CALCULATION

F-3.1 Acid insoluble ash (on moisture-free basis), percent by mass

$$= \frac{100 (W_2 - W)}{W_1 - W}$$

where

W_2 = the lowest mass in g of the dish with the acid insoluble ash,

W = mass in g of the empty dish, and

W_1 = mass in g of the dish with the dried material (*see* W_1 in **E-2.1**).

---

*Specification for hydrochloric acid (*revised*).

APPENDIX G

[*Table* 1, *Item* (vii)]

DETERMINATION OF FLUORINE

G-1. APPARATUS

G-1.1 Distillation Flasks

G-1.2 Nessler Tubes — of 50 ml capacity.

G-1.3 Microburette

G-2. REAGENTS

G-2.1 Lime-Water Freed from Fluorine — Dissolve lime in an excess of perchloric acid. Boil for 15 minutes. Dilute. Cool and neutralize with fluorine-free sodium hydroxide. Filter through a Buchner funnel and wash. Make a saturated solution in distilled water using the lime thus freed from fluorine.

G-2.2 Perchloric Acid Solution — 60 to 70 percent (*m/v*). Heat some quantity for an hour or longer at 140 to 150°C.

G-2.3 Silver Perchlorate Solution — one percent (*m/v*). Prepare by adding sufficient sodium hydroxide solution to a solution of silver nitrate to cause precipitation. Filter and wash the precipitate with water. Dissolve the precipitate in perchloric acid and dilute.

G-2.4 Sodium Hydroxide Solution — 0·05 N.

G-2.5 Alizarin Indicator Solution — Dissolve 0·02 g of sodium alizarin sulphonate in water and make up the volume to 100 ml.

G-2.6 Dilute Hydrochloric Acid — 0·05 N.

G-2.7 Buffer Solution — Dissolve 0·10 g of hydroxylamine hydrochloride in water and make up the volume to 100 ml.

G-2.8 Thorium Nitrate Solution — Dissolve 0·5 g of hydrated thorium nitrate [$Th(NO_3)_4, 12H_2O$] in distilled water and make up the volume to one litre.

G-2.9 Standard Fluorine Solution — Dissolve 2·211 g of sodium fluoride in water and make up the volume to one litre in a graduated flask. Pipette out 10 ml of this solution into a one-litre graduated flask and make up the volume. This solution contains 0·01 mg of fluorine per millilitre.

G-3. PROCEDURE

G-3.1 Weigh accurately about 5 g of the material in a platinum dish. Moisten with lime-water. Dry on a water-bath and ignite in a muffle furnace at about 550°C. When the ashing is complete, cool and transfer to a distillation flask, washing it with water. Dissolve the residual ash in 10 to 15 ml of perchloric acid solution and transfer to the same distillation flask. Add sufficient silver perchlorate solution to bring about complete precipitation. Steam-distil at 132 ± 3°C into another flask containing 2 ml of the sodium hydroxide solution. Collect about 150 ml of the distillate. Transfer the distillate to a 200-ml graduated flask and make up the volume to the mark with water.

G-3.2 Transfer a suitable aliquot of the test solution containing 10 to 30 μg of fluorine to a Nessler tube. Add one millilitre of alizarin indicator solution. Take the same quantity of the indicator solution in another Nessler tube. If necessary, the alkali in the test solution is neutralized with a drop or two of dilute hydrochloric acid. To each tube; add one millilitre of the buffer solution, 2·0 ml of dilute hydrochloric acid and dilute to about 45 ml. The colour of the solutions should be straw-yellow. Add the thorium nitrate solution from a microburette to the test solution until a permanent slight pink colour appears (0·5 to 2·0 ml of the thorium nitrate solution would be required). Add an equal volume of the thorium nitrate solution to the other Nessler tube. Adjust the colour of the solution in this Nessler tube by adding the standard fluorine solution from a microburette to the same intensity as the colour of the test solution. When the colours in both the tubes match the amount of fluorine in the standard fluorine solution added is equal to the amount of fluorine present in the aliquot of the test solution. From this calculate the amount of fluorine present in 100 g of the material on moisture-free basis.

APPENDIX H

[*Table* 1, *Item* (viii)]

DETERMINATION OF SPORES OF *BACILLUS ANTHRACIS* AND *CLOSTRIDIUM* SPP.

H-1. DETECTION OF *BACILLUS ANTHRACIS*

H-1.1 Method — The method consists of isolation of the organisms, microscopic examination, study of the biochemical reactions and the biological test.

H-1.2 Reagent

H-1.2.1 *Sterile Sodium Chloride Solution* (*Normal Saline*) — 0·9 percent (*m/v*).

H-1.3 Medium

H-1.3.1 *Blood Agar Medium* — Prepare as follows:

Add sterile defibrinated blood (5 to 10 percent by mass), collected aseptically from a healthy horse or ox, to nutrient agar that has been melted and cooled to 48°C. Mix well and pour into sterile plates with aseptic precautions before flame and allow to set. Incubate for 15 hours at 37°C.

H-1.4 Procedure for Isolation and Examination of the Organism — Shake about 100 g of the material with 1 00J ml of sterile sodium chloride solution. Decant the supernatant liquid. Centrifuge and suspend the packed sediment in about 3 ml of the sterile sodium chloride solution. Heat for 10 minutes at 70°C in a water-bath. Streak on at least three blood agar plates from this suspension and incubate at 37°C for 24 hours. Examine the characteristic colonies of *B. anthracis*. The colonies are circular and disc-like, white granular, about 3 mm in diameter, showing a ground-glass appearance and a wavy margin with only a very slight zone of hæmolysis. On being examined microscopically, the colonies will consist of non-motile, gram-positive, rod-shaped organisms, 3 to 8 μm × 1 to 1·2 μm in size, straight or slightly curved with truncated ends arranged characteristically in very long, segmented, parallel or inter-woven chains. The organism ferments salicin slowly with the production of acid alone, reduces methylene blue weakly and shows a weak lecithinace reaction.

H-1.5 Procedure for Biological Test — Inject subcutaneously about one millilitre of the heated suspension into two guinea pigs. If anthrax spores are present in the material, the guinea pigs will die in 12 to 48 hours showing hæmorrhagic local exudate and enlargement of spleen on post mortem. Capsulated organisms will be found in stained smears made from blood and also from the œdema fluid at the site of injection spleen, liver and peritoneal fluid. The capsule is demonstrated easily by staining the preparation with Lœffler's alkaline methylene blue stain.

H-2. DETECTION OF *CLOSTRIDIUM BOTULINUM*

H-2.1 Medium — Either the peptic digest of beef heart or the corn steep casein glucose broth to which 0·2 percent of soluble starch has been added may be used.

H-2.1.1 *Peptic Digest of Beef Heart* — Prepare as follows:

Add 600 g of ground and defatted beef heart to 2 litres of distilled water. Adjust the *p*H to 1·8 to 2·0 with hydrochloric acid. Add 2 g of pepsin and mix thoroughly. Incubate overnight at 45 to 50°C. Filter, neutralize with sodium hydroxide and filter again. Add peptone (one percent) and glucose (0·5 percent). Dispense and sterilize by steaming for 90 minutes in a steam sterilizer.

H-2.1.2 *Corn Steep Casein Glucose Borth* — Prepare as follows:

Add 3 g of casein to 50 ml of distilled water and adjust the *p*H to 10·5 to 11·5. Add 5 g of glucose to a volume of corn steep liquor equivalent to 5 g of total solids and dilute to 800 ml with distilled water. To this, add the casein suspension and adjust the *p*H to 7·3. Add distilled water to make up the volume to one litre. Dispense and sterilize by autoclaving.

H-2.2 Procedure — Inoculate about one gram of the material into each of several conical flasks containing about 250 ml of the medium. Heat at 80°C for 30 minutes in a water-bath and incubate the different flasks at different temperatures (20°, 25°, 35° and 37°C) anærobically for 10 days. Inject 2 ml of the bacteria-free culture filtrate from each flask intraperitoneally into two guinea pigs (0·2 ml in the case of mice). Observe for the development of characteristic symptoms which are: hypersecretion of saliva in a day or so, shallow breathing, dyspnœa, muscular paralysis, dilated pupils, pendulous abdomen, prostration and death in 1 to 4 days. In rare cases, death may be delayed to 1 to 2 weeks with a gradual loss of mass and increasing prostration.

NOTE — No suspected organism should be identified as *Clostrdium botulinum* unless it produces the characteristic toxin in the suitable medium.

H-3. DETECTION OF *CLOSTRIDIUM CHAUVŒI* AND *CLOSTRIDIUM SEPTICUM*

H-3.1 Procedure — Mix about 100 g of the material with about 1 000 ml of the sterile sodium chloride solution. Decant the supernatant fluid and centrifuge. Suspend the packed sediment in about 3 ml of the sterile sodium chloride solution. Heat at 70°C for 20 minutes in a water-bath. Inoculate one millilitre of the heated suspension intramuscularly into each of two healthy guinea pigs along with 0·5 ml of a freshly prepared sterile calcium chloride solution (3 percent) (*m/v*). Observe for 1 to 4 days within which time the animals will die of gas gangrene of the muscles at the site of injection in case the spores of *Clostridium chauvæi* or *Clostridium septicum* or both should be present. Post-mortem should be conducted immediately after the animal dies and the liver impression preparation will reveal filamentous forms if the cause of death is *Clostridium septicum*. In addition, streak the heated sample on several freshly prepared blood agar plates and incubate anærobically at 37°C for 3 to 5 days. *Clostridium chauvæi*, if present, will develop in the form of irregularly round colonies 3 to 6 mm in diameter, effuse with entire or rhizoid edge. There will be no definite hæmolytic zone but the plate will be cleared slightly around the colonies. *Clostridium septicum* colonies will be larger, about 10 mm in diameter, irregularly round, effuse and having a cigarette-in-water appearance. Hæmolysis will be of the *beta* type with prolonged incubation.

APPENDIX J

(*Clause* 4.1)

SAMPLING OF BONE-MEAL

J-1. GENERAL REQUIREMENTS OF SAMPLING

J-1.0 In drawing, preparing, storing and handling test samples, care should be taken that the properties are not affected. The following precautions and directions shall be observed.

J-1.1 Samples shall be taken in a protected place, not exposed to damp air, dust or soot.

J-1.2 The sampling instrument shall be clean, dry and sterile.

J-1.3 Precautions shall be taken to protect the samples, the material being sampled, the sampling instrument and the containers for samples from adventitous contamination.

J-1.4 The samples shall be placed in clean, dry and sterile glass containers. The sample containers shall be of such size that they are almost completely filled by the samples.

J-1.5 Each container shall be sealed air-tight with a stopper or a suitable closure in such a way that it could not be opened and resealed without detection, after filling and marked with full details of sampling, date of sampling, date of manufacture, batch number, name of the manufacturer, and other important particulars of the consignment.

J-1.6 Samples shall be stored in such a manner that there is no deterioration of the material.

J-1.7 Sampling shall be done by a person agreed to between the purchaser and the vendor and, if desired by any of them, in the presence of the purchaser (or his representative) and the vendor (or his representative).

J-2. SCALE OF SAMPLING

J-2.1 Lot — All the containers in a single consignment of the material drawn from a single batch of manufacture shall constitute a lot. If a consignment is declared to consist of different batches of manufacture, the batches shall be grouped separately and the containers in each group shall constitute a separate lot.

J-2.1.1 Samples shall be tested for each lot for ascertaining conformity of the material to the requirements of this specification.

J-2.2 The number of containers to be selected from a lot shall depend on the size of the lot and shall be in accordance with col 1 and 2 of Table 2.

TABLE 2 NUMBER OF CONTAINERS TO BE SELECTED FOR SAMPLING

LOT SIZE (N) (1)	NO. OF CONTAINERS TO BE SELECTED FOR SAMPLING (n) (2)
2 to 15	2
16 " 40	3
41 " 65	4
66 " 110	5
111 " 180	6
181 " 300	7
301 " 450	8
451 " 600	9
601 " 800	10

J-2.3 The containers to be selected for sampling shall be chosen at random from the lot and for this purpose, a random number table as agreed to between the purchaser and the vendor shall be used. If such a table is not available, the following procedure shall be adopted:

> Starting from any container, count 1, 2, 3, etc, up to r and so on in a systematic manner. Every rth container shall be withdrawn, r being the integral part of N/n, where N is the total number of containers in the lot and n the number of containers to be selected.

J-3. TEST SAMPLES AND REFEREE SAMPLES

J-3.1 Preparation of Individual Samples — Draw with a appropriate sampling instrument equal quantities of the material from different parts of each container selected according to Table 2. The total quantity of material drawn from each container shall be not less than 1·5 kg. Mix all the portions of the material drawn from the same container thoroughly. Take out about 0·75 kg of the material and divide it into three equal parts. Each portion thus obtained shall constitute the test sample representing that particular container and shall be transferred immediately to clean, dry and sterile container and sealed air-tight. These shall be labelled with the particulars given in J-1.5. The individual samples so obtained shall be divided into three sets in such a way that each set has a test sample representing each container selected. One of the sets shall be for the purchaser another for the vendor and the third for the referee.

J-3.2 Preparation of Composite Samples — From the mixed material from each selected container remaining after the individual samples have been taken, equal quantities of the material from each container shall be taken and mixed together so as to form a composite sample weighing not less than 0·75 kg. This composite sample shall be divided into three equal parts and transferred to clean dry and sterile containers which shall be labelled with the particulars given in **J-1.5** and sealed air-tight. One of these samples shall be for the purchaser, another for the vendor and the third for the referee.

J-3.3 Referee — Referee samples shall consist of a set of test samples (*see* **J-3.1**) and a composite sample (*see* **J-3.2**) and shall bear the seals of the purchaser and the vendor and shall be kept at a place agreed to between the two.

J-4. TESTING OF SAMPLES

J-4.1 Tests for phosphorus shall be conducted individually on each of the samples constituting the set of test samples (*see* **J-3.1**).

J-4.2 Tests for the remaining characteristics specified in **2.2** shall be conducted on the composite sample (*see* **J-3.2**).

J-5. CRITERION FOR CONFORMITY

J-5.1 A lot shall be considered as conforming to the specification, when:

a) each of the test results for phosphorus satisfies the requirement as specified in **2.2**, and

b) the test results on the composite sample satisfy the other requirements as specified in **2.2**.

J-5.2 If one or more test results do not satisfy the requirement for phosphorus, the following procedure shall be adopted for determining conformity of the material for phosphorus:

Calculate the mean and range of the test results as follows:

$$\text{Mean } (\bar{X}) = \frac{\text{Sum of the test results}}{\text{Number of the test samples}}$$

Range (R) = Difference between the maximum and the minimum values of the test results

If $\bar{X} - 0{\cdot}4\,R$ greater than or equal to 15·0, the lot shall be considered as conforming to the specification in regard to phosphorus.

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